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Induction of Human Granulocyte Chemiluminescence by Bacterial Lipopolysaccharides

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Bacterial lipopolysaccharides (LPS) have been reported to influence the oxidative response of human polymorphonuclear leukocytes (PMN). However, results sometimes conflict. In the present study, we demonstrated that activation of human PMN by LPS depends on the class (smooth [S] or rough [R]) to which the LPS belongs. Lucigenin-dependent chemiluminescence was used to assay oxygen radical production. Twenty different S- and R-form LPS and free lipid A were tested in concentrations of 0.01 to 100 μg/ml. S-form LPS activated PMN only at maximal concentrations and to a low extent. R-form LPS and free lipid A were potent inducers of granulocyte chemiluminescence even at a concentration of 0.1 μg/ml. The results indicated that R-form LPS are very effective in inducing granulocyte chemiluminescence, whereas true S-form preparations are inactive. It is not known at present whether this higher activity is due to a more lipophilic character of R-form LPS or whether the presence of the O polysaccharide in S-form LPS exerts an inhibitory effect on their action on granulocytes.

Several researchers showed that lipopolysaccharides (endotoxin; LPS) can influence the oxidative metabolism of human neutrophils. It could be shown recently that LPS significantly enhanced the hexose monophosphate shunt activity in human polymorphonuclear leukocytes (PMN) (5). Proctor (18) found that endotoxin is able to depress neutrophil bactericidal activity while enhancing nitroblue tetrazolium reduction and hexose monophosphate shunt activity. However, human PMN did not show chemiluminescence under stimulation with LPS or lipid A. Wilson et al. (22) reported that LPS failed to augment oxygen consumption, superoxide generation, or chemiluminescence and suggested that bacterial LPS do not directly activate neutrophil oxidative metabolism. Guthrie et al. (12) reported that minimal concentrations of LPS primed neutrophils for enhanced release of oxygen metabolites. Henricks et al. (14) investigated the effect of different LPS preparations on human PMN. They found that *E. coli* LPS diminish or enhance different cell functions of human PMN. One rough (R)-form LPS preparation and free lipid A induced PMN to generate superoxide and chemiluminescence; however, they lowered phagocytic, chemotactic, and metabolic activities in PMN. Smooth (S)-form LPS had only marginal activity. Oxygen radical scavengers, when present during the experiments, prevented the induced defects in PMN function, and the investigators hypothesized that the lipid A portion of LPS is toxic for PMN because of induction of toxic oxygen species. Thus, the results of the different studies are contradictory. These differences may be due to the different endotoxin preparations used. The present study was carried out to investigate whether LPS can stimulate human PMN directly. Furthermore, the question was raised whether a correlation exists between the chemical structure of the tested preparations and the resulting degree of activation. Activation of human PMN was assessed by using lucigenin-dependent chemiluminescence (1, 2) as a sensitive measure of the release of oxygen radicals. It was shown that induction of granulocyte chemiluminescence by LPS is generally a property shared by R-form preparations and that the low activity of S-form LPS is due to the presence of R-form molecules that are always present in variable amounts in such preparations.

MATERIALS AND METHODS

Ficoll Hypaque solution was obtained from Pharmacia, Freiburg, Federal Republic of Germany. Lucigenin and the *Limulus* amoebocyte lysate assay were obtained from Sigma, München, Federal Republic of Germany. Culture media were from Biochrom Seromed, Berlin, Federal Republic of Germany. Since trace concentrations of bacterial LPS were able to prime neutrophils (13), all materials, solutions, and media were tested for the presence of trace concentration of LPS by the *Limulus* amoebocyte lysate assay.

LPS. S-form LPS were isolated from parent bacteria by the phenol-water procedure (21), R-form LPS were isolated by the phenol-chloroform-petroleum ether isolation method (10). Uniform salt forms of the various preparations used were prepared after electrodialysis of the LPS as described earlier (9). In this way, preparations in low (triethylammonium salt), medium (Na^+ salt), and high (Ca^{2+} form) degrees of aggregation were obtained (9). R-form LPS were tested as low-molecular-weight triethylammonium salts. The LPS preparations were freshly diluted from stock solutions before testing. Free lipid A was prepared by acid hydrolysis of *Escherichia coli* 515 (Re) LPS in acetate buffer (0.1 M, pH 4.6) at 100°C for 2 h (3). It was converted to the soluble triethylammonium form as described earlier (10).

Fractionation of *S. abortusequi* LPS into pure S-form and R-form enriched fractions. Details of the fractionation procedure will be described elsewhere (C. Galanos and T. Komuro, manuscript in preparation). Briefly, *Salmonella abortusequi* LPS in the triethylammonium salt, prepared as described in reference 9, was extracted several times by refluxing in a mixture of chloroform-methanol-water; each time, the soluble and insoluble fractions were separated by centrifugation. Analysis of the different soluble fractions and the final residue by sodium dodecyl sulfate-polyacrylamide

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TABLE 1. Effect of S-form LPS and lipid A on granulocyte chemiluminescence

Source of LPS	Mean (± SEM) chemiluminescence (% of control) at an LPS concn (μg/ml) of[a]:				
	0.01	0.1	1.0	10.0	100.0
E. coli O73	122 ± 21	110 ± 14	137 ± 24	207 ± 43	171 ± 27
E. coli O31	89 ± 14	97 ± 9	128 ± 27	220 ± 43	194 ± 72
S. michigan	99 ± 1	96 ± 14	134 ± 10	208 ± 19	175 ± 25
E. coli O75	101 ± 12	115 ± 8	125 ± 8	126 ± 25	124 ± 18
S. enteritidis	84 ± 14	107 ± 19	121 ± 15	114 ± 32	326 ± 73
S. minnesota	109 ± 2	127 ± 21	159 ± 17	157 ± 10	434 ± 164
S. ruiru	110 ± 10	104 ± 15	109 ± 37	128 ± 14	190 ± 14
S. tel-aviv	132 ± 8	139 ± 8	118 ± 29	218 ± 54	290 ± 36
S. typhimurium	92 ± 26	107 ± 32	121 ± 38	128 ± 40	373 ± 123
S. abortusequi	88 ± 7	85 ± 6	92 ± 21	161 ± 42	410 ± 36

[a] Medium control was taken as 100% as described in Materials and Methods. The mean response induced by PMA (25 ng/ml) was 6,691 ± 204%. The data are from three experiments on PMN of three different donors.

gel electrophoresis revealed that the insolubilized fraction consisted only of high-molecular-weight bands (designated the S-form fraction). Bands corresponding to R-form LPS or S-form LPS of medium molecular weight were absent. The fraction obtained by solubilization in the extraction mixture was devoid of high-molecular-weight bands; it consisted only of bands corresponding to R-form LPS and S-form LPS of low molecular weight (designated the R-form enriched fraction). The S-form fraction and the R-form enriched fraction were investigated in the present study for their chemiluminescence-inducing properties in granulocytes.

Isolation of PMN. Human PMN were isolated from 20 ml of venous blood of healthy blood donors as described previously (15). Briefly, separation was performed by Ficoll gradient centrifugation and three 30-s cycles of hypotonic lysis with distilled water followed by addition of an equal volume of 1.8% NaCl, and PMN were finally suspended to a density of 5×10^6 per ml in phosphate-buffered saline, pH 7.2, containing 1 mM calcium, 0.5 mM magnesium, and 2 mM lucigenin. Cells were 98% PMN as judged by Pappenheim stain and more than 95% viable as tested by trypan blue exclusion. Samples (200 μl) containing 10^6 cells each were placed into unsealed polystyrene luminescence tubes (Lumacuvette; Abimed, Düsseldorf, Federal Republic of Germany) and stored at 4°C for a maximum of 6 h before use.

Chemiluminescence measurements. Cells were warmed to 37°C for 15 min before the measurements. Chemiluminescence measurements were performed in a six-channel Biolumat LB 9505 (Berthold, Wildbad, Federal Republic of Germany) interfaced via an Apple IIE computer to an Epson RX80 graphic printer. Measurements were made at 37°C. Samples (20 μl) of the different LPS dilutions and distilled water as a control were added simultaneously to the six channels of the Biolumat, and 45-min integral counts were measured. Usually, each LPS preparation was tested on the PMN of three different donors. For calculation of the results, integral counts induced by the medium control were set at 100%. All values of one run were calculated in relation to the control. As a positive control, phorbol myristate acetate (PMA) was used at a concentration of 25 ng/ml to assess the oxidative response of PMN. In different experiments, the response to PMN ranged from 6,089 to 7,240% of the control. The mean of these values ± the standard error of the mean (SEM) is given in the legend of each table.

RESULTS

In the present study, we tested the effects of different S- and R-form LPS and free lipid A on granulocyte chemiluminescence. The different preparations were tested in a concentration range of 0.01 to 100 μg/ml. All S-form LPS preparations induced only minimal effects at concentrations under 10 μg/ml (Table 1). Only at higher concentrations did some preparations (*S. minnesota* and *S. abortusequi*) induce a low but significant increase in the chemiluminescence response. In contrast to S-form LPS, R-form LPS (Table 2) significantly stimulated the oxidative response of PMN by itself even at a concentration of 0.1 μg/ml. Of the different R-form preparations tested, those belonging to the Rd class, *S. minnesota* Rz (Rd_1P^+), *S. minnesota* R7 (Rd_1P^-), and *S. minnesota* R3 (Rd_2P^-), although active, had consistently lower activity than did preparations of the other R classes. The reason for this is not known at the moment. Free lipid A was very potent, being comparable in its activity to the more active R-form preparations. This finding makes it evident

TABLE 2. Effect of R-form LPS and lipid A on granulocyte chemiluminescence

Source of LPS (form)	Mean (± SEM) chemiluminescence (% of control) at an LPS concn (μg/ml) of[a]:				
	0.01	0.1	1.0	10.0	100.0
S. minnesota R60 (Ra)	132 ± 20	508 ± 209	838 ± 212	848 ± 39	2,073 ± 594
E. coli EH100 (RII)	159 ± 24	492 ± 21	545 ± 44	1,120 ± 505	1,982 ± 320
S. minnesota R345 (Rb)	122 ± 21	355 ± 123	655 ± 192	842 ± 99	2,164 ± 709
S. minnesota R5 (Rc)	90 ± 16	231 ± 40	327 ± 73	411 ± 55	907 ± 30
S. minnesota RZ (d_1P^+)	154 ± 11	230 ± 27	286 ± 40	279 ± 2	630 ± 168
S. minnesota R7 (Rd_1P^-)	127 ± 5	154 ± 8	221 ± 72	251 ± 84	403 ± 143
S. minnesota R3 (Rd_2)	155 ± 45	305 ± 70	436 ± 108	412 ± 133	448 ± 221
S. minnesota R595 (Re)	107 ± 3	529 ± 261	1,097 ± 347	1,188 ± 517	1,127 ± 555
E. coli F515 (Re)	101 ± 4	304 ± 85	553 ± 108	645 ± 132	1,176 ± 170
Lipid A	246 ± 38	533 ± 41	994 ± 76	1,625 ± 264	2,322 ± 183

[a] Medium control was taken as 100% as described in Materials and Methods. The mean response induced by PMA (25 ng/ml) was 6,691 ± 204%. The data are from three experiments on PMN of three different donors.

that lipid A is the part of the LPS molecular responsible for induction of chemiluminescence in granulocytes. Since the high activity of R-form preparations might be due to a higher degree of aggregation, the activity of *S. abortusequi* LPS in the Ca^{2+}, Na^{+}, and triethylammonium forms was tested. All three salt forms showed a low activity comparable to that of the starting preparation (data not shown), indicating that the presence or absence of chemiluminescence was not dependent on a differential degree of aggregation.

Kinetics of the chemiluminescence response. In Fig. 1, typical kinetics of chemiluminescence induction in human granulocytes is shown with free lipid A. The response begins about 5 min after addition of lipid A and reaches maximum values at 20 min. Thereafter, it decreases, with control values being reached 60 min after the start. The kinetics for 1 and 0.1 μg of lipid A are identical, the two concentrations varying only in their peak maxima. The above kinetics obtained for lipid A are typical for all preparations used in this study.

Effect of fractionated *S. abortusequi* LPS on granulocyte chemiluminescence. The chemiluminescence-inducing properties of the S-form fraction and the R-form enriched fraction obtained by fractionation of *S. abortusequi* LPS are shown in Table 3 and compared with those of the starting preparation. The starting LPS was of only low activity, which was expressed at the highest concentration (100 μg/ml). In contrast, high activity was seen with the R-form enriched fraction, with which significant chemiluminescence was obtained even with 0.1 μg/ml. The S-form fraction was completely inactive up to 100 μg/ml. From these results, it was concluded that the low activity expressed by S-form LPS is due to low amounts of R-form material present.

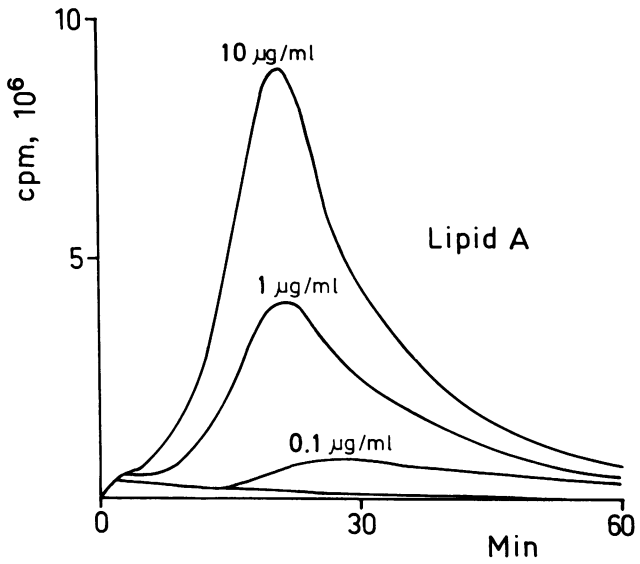

FIG. 1. Typical time course of the chemiluminescence response by PMN incubated with different concentrations of free lipid A. The peak maximum is comparable to that induced by phorbol-myristate acetate (10 ng/ml).

DISCUSSION

Induction of chemiluminescence in human granulocytes proceeded with R-form LPS and free lipid A and was nearly absent with S-form LPS. In most cases, amounts of 0.1 μg of R-form LPS per ml sufficed to induce significant activation, whereas S-form preparations were not active or only of low activity in concentrations up to 100 μg/ml. We therefore concluded that induction of granulocyte chemiluminescence by LPS is a property of R-form LPS, S-form preparations being virtually devoid of this activity. The present data showed that the findings of Henricks et al. (14) on the induction of chemiluminescence obtained with one S- and one R-form LPS are valid generally for S- and R-form LPS. The low activity seen with some S-form LPS is due to the variable amounts of R-form LPS that are usually present in such preparations, as is evident after fractionation.

The high activity of free lipid A seen in this study showed that, as for most biological activities of LPS, for induction of granulocyte chemiluminescence, lipid A is the part of the LPS molecule responsible for the activity. It is not known at the moment why the activity of lipid A is not expressed in S-form LPS. The more than 100-fold lower activity of S-form LPS compared with the R-form preparations seen here cannot be explained solely by the lower amounts of lipid A they contain. Other reasons, such as insufficient hydrophobicity or an inhibitory effect of the O-polysaccharide chains, may play a role. Both classes of LPS are usually of comparable activity in their lethal properties, e.g., in mice sensitized by D-galactosamine (8) S- and R-form LPS are also very similar in their pyrogenic and Shwartzman reaction-inducing properties (7). However, in some respects R-form LPS may differ qualitatively and quantitatively in activity from S-form preparations. R-form LPS are cleared faster from the blood and, in contrast to S-forms, which are taken up exclusively by Kupffer cells, have direct access to hepatocytes (6). A preferential interaction of R-form LPS with hepatocytes has also been demonstrated in vitro (19). Distinct differences between S- and R-form LPS also exist in their abilities to interact with the complement system. Activation of the complement cascade by S-form LPS proceeds via the alternate pathway, and that by R-form LPS occurs via the classical pathway (4, 16, 17). A number of R-form LPS belonging mainly to classes Ra and Rb were long ago recognized as having little effect on the complement system (11). Very recently, activation of the classical pathway of complement activation was shown to be restricted to lipid A and Re-form LPS (20).

In this study, a correlation between the granulocyte-activating properties of the different LPS preparations and their ability to activate the complement system was not evident. This was especially true of *S. minnesota* R60 (Ra) and R345 (Rb) LPS, which were very active in the present system but have always been found to be weak activators of the complement system.

It is often difficult to recognize differences between S- and R-form LPS clearly because S-form LPS almost always contain variable amounts of R-form molecules, which cause overlapping in activity. This was clearly shown in the present study, in which *S. abortusequi* S-form LPS exhibited low activity; however, after fractionation the portion enriched in R-form LPS was highly active and indistinguishable from R-form preparations.

The reason for the difference in activity between S- and R-form LPS is not known at the moment. The present results show at least that this difference is not due to a different degree of aggregation of the preparations since the activity of one and the same LPS was not affected by the salt form and, therefore, the degree of aggregation. One possible reason for the activity of R-form LPS may be their higher lipophilic properties due to the high concentration of lipid A in the

TABLE 3. Effect of fractionated *S. abortusequi* S-form LPS on granulocyte chemiluminescence

LPS	Mean (± SEM) chemiluminescence (% of control) at an LPS concn (μg/ml) of[a]:				
	0.01	0.1	1.0	10.0	100.0
Original LPS	88 ± 7	85 ± 6	92 ± 21	161 ± 42	410 ± 36
S-form fraction	104 ± 12	106 ± 22	110 ± 23	97 ± 24	127 ± 13
R-form enriched fraction	168 ± 33	504 ± 128	825 ± 186	1,201 ± 284	3,294 ± 465

[a] Medium control was taken as 100%. The mean response induced by PMA (25 ng/ml) was 6,691 ± 204%. The data are from three experiments on PMN of three different donors.

molecule, which may render them more phagocytosable by PMN than are S-form LPS.

The parameter of granulocyte activation by LPS used in this study, chemiluminescence, is believed to be dependent on generation of reactive oxygen radicals produced by activation of a membrane NAD(P)H oxidase (1). Oxygen radicals are believed to possess bactericidal properties but also, because of their toxicity, to cause tissue damage during inflammation. Understanding of the activation of granulocytes by endotoxin will enable a better estimation of their role in endotoxic reactions.

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